

Original article

DOI: 10.1515/aiht-2016-67-2795

Protective effects of quercetin and vitamin C against nicotine-induced toxicity in the blood of Wistar rats

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[Received in March 2016; CrossChecked in March 2016; Accepted in November 2016]

Nicotine is a potential inducer of oxidative stress, through which it can damage numerous biological molecules. The aim of our study was to investigate the prooxidative effects of nicotine and protective (additive or synergistic) effects of quercetin and vitamin C in the blood of experimental animals, to determine whether the combination of these antioxidants might be beneficial for clinical purposes. Wistar albino rats were receiving intraperitoneal nicotine injection (0.75 mg kg⁻¹ per day) or saline (control group) or nicotine plus quercetin (40 mg kg⁻¹ per day) and vitamin C (100 mg kg⁻¹ per day) for three consecutive days. On day 4, we determined their blood lipid profile, liver enzymes, oxidative stress parameters, and antioxidative system parameters. Compared to untreated control, nicotine significantly increased total cholesterol, LDL-cholesterol, triglycerides, liver enzymes (alanine transaminase, aspartate transaminase, and lactate dehydrogenase) and oxidative stress parameters (superoxide anion, hydrogen peroxide, and lipid peroxide) and decreased HDL-cholesterol, glutathione, and superoxide dismutase/catalase activity. Quercetin + vitamin C reversed these values significantly compared to the nicotine alone group. Our results confirm that nicotine has significant prooxidative effects that may disrupt the redox balance and show that the quercetin + vitamin C combination supports antioxidant defence mechanisms with strong haematoprotective activity against nicotine-induced toxicity. In practical terms, this means that a diet rich in vitamin C and quercetin could prevent nicotine-induced toxicity and could also be useful in the supportive care of people exposed to nicotine.

KEY WORDS: *antioxidant defence; erythrocytes; lipid profile; oxidative damage; reactive oxygen species; tobacco*

Nicotine, the primary addictive component of cigarette smoke, is rapidly absorbed by the circulatory system and most of it is metabolised in the liver (1). Previous *in vivo* studies have shown that chronic use of nicotine disrupts the balance between the prooxidants and antioxidants in the circulation of experimental rats (2). Nicotine triggers the production of free radicals and reactive oxygen species (ROS), including superoxide anion radical (O₂^{•-}), hydrogen peroxide (H₂O₂), and hydroxyl radical (•OH), which overwhelm the antioxidative defence system (AOS) and eventually generate oxidative stress (3-6).

Red blood cells are a good indicator of oxidative stress, as their components, haemoglobin and polyunsaturated fatty acids, are susceptible to it (7), which is why they contain antioxidants for efficient defence. These components are non-enzymatic [such as vitamins C and E, and glutathione (GSH)] and enzymatic [superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px)] (8). The defence system can be improved by the consumption of fruits and vegetables rich in antioxidants, including vitamin C and flavonoids - polyphenolic compounds common in fruits, vegetables, and some types of tea and wine (9).

One such flavonoid found in abundance in red kidney beans, grapes, red wine, apples, tea, onions, and broccoli is quercetin. Its consumption in everyday diet is too low for a clinically meaningful protective effect but it can easily be increased with quercetin-rich food and supplements (10), whereas its bioavailability increases if consumed with other flavonoids, vitamins C and E, or catechins (11-13).

Considering that Muthukumaran et al. (14) have already demonstrated the antioxidant properties of quercetin alone against nicotine-induced oxidative stress in rats, the aim of our study was to see how effective the combination of quercetin and vitamin C could be, especially as this combination has never been studied before. By establishing conclusive evidence of the combination's effectiveness against nicotine-induced toxicity, we hoped to see whether this combination could find clinical application. Our secondary aim was to get an insight into the possible underlying mechanisms of (inter)action between nicotine and the combination of antioxidants.

MATERIALS AND METHODS

Chemicals

All reagents and chemicals used in this study were of analytical grade or higher purity, obtained from Sigma

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Chemical Co. (St. Louis, MO, USA) and Merck (Darmstadt, Germany). The solutions were prepared with double-distilled water.

Experimental design

All the experimental procedures were approved by the University Ethics Committee for Animal Experimentation. We used 18 male albino *Wistar* rats, about 2 months old, weighing 200–220 g to exclude possible protective effects of oestradiol against provoked oxidative stress (15, 16). Before the experiments began, the animals were housed in plastic cages under standard laboratory conditions (temperature 22 ± 2 °C; 12:12 h light:dark cycle) for at least one week to adapt. Tap water and commercial standard rodent laboratory diet were available *ad libitum* for the duration of the experiments. The rats were randomly divided into three groups ($n=6$ per group) and treated for three days, as follows: the control group was receiving intraperitoneal (*i.p.*) injections of isotonic saline [0.1 mL kg^{-1} body weight (b.w.)]; the nicotine group was receiving nicotine only [0.75 mg kg^{-1} (b.w.) per day *i.p.*]; and the nicotine + quercetin + vitamin C group was receiving nicotine as described above with quercetin [40 mg kg^{-1} (b.w.) per day *i.p.*] and vitamin C [100 mg kg^{-1} (b.w.) per day *i.p.*]. These doses were based on literature data (4, 17–21) and correspond to the human daily dietary intake of quercetin and vitamin C enriched food and the daily nicotine intake in people who smoke 10–20 cigarettes per day (2, 4).

Twenty-four hours after the last dose, the animals were anaesthetised with ether and decapitated. Blood samples for all analyses were collected on the day of sacrifice between 8 a. m. and 10 a. m. to avoid variations related to the circadian rhythm.

Analytical procedures

For the medium we selected rat blood as the best substrate. The blood samples were collected in tubes with anticoagulant (K-EDTA) and without one. Biochemical parameters were measured on the day of sacrifice. Plasma for the other analyses was separated by centrifugation at 1000 g ($+4$ °C) for 10 min. The erythrocytes were washed three times with an equal volume of cold saline (0.9 %, v/v). One millilitre of the washed erythrocytes was lysed with 3 mL of dH_2O (1:3, v/v) at 0 °C for 30 min. All samples were extracted from the lysates and stored at -20 °C for no longer than 7 days. ROS analysis was completed within 1–2 h after extraction. Lipid peroxides (LPO) and GSH were measured within 6 h after extraction. Antioxidant enzyme activity in erythrocyte lysate was measured following the method described by McCord and Fridovich (22).

Biochemical analysis

Blood samples in non-anticoagulant tubes were centrifuged at 1000 g ($+4$ °C) for 15 min to obtain serum

for measurements. We analysed the serum for total cholesterol, high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL), triglycerides (TG) (expressed in mmol L^{-1}), and enzyme activity [alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH, expressed in U L^{-1})] with a C 8000 Architect autoanalyser (Wiesbaden, Germany) using standard diagnostic colourimetric kits (Abbott Laboratories, Abbott Park, IL, USA).

Oxidative stress parameters

Erythrocyte ROS concentrations were determined as follows: half the volume of 3 mol L^{-1} perchloric acid and 2 volumes of 20 mmol L^{-1} EDTA were added to one volume of the lysate. After extraction on ice and centrifugation on 1000 g ($+4$ °C) for 10 min, the extracts were neutralised with 2 mol L^{-1} of K_2CO_3 . The spectrophotometric determination of $\text{O}_2^{\bullet-}$ was based on the reduction of nitroblue tetrazolium in the presence of $\text{O}_2^{\bullet-}$ (23). The determination of H_2O_2 was based on the oxidation of phenol red in the presence of horseradish peroxidase as catalyst (24). These concentrations are expressed in $\mu\text{mol L}^{-1}$ of erythrocytes.

Lipid peroxides in the haemolysate were determined using the method described by Ohkawa et al., (25), based on the reaction of lipid peroxidation products (malondialdehydes) with thiobarbituric acid reactive substances (TBARS). Haemolysate samples were extracted with 28 % trichloroacetic acid and centrifuged at 1000 g for 10 min. For colour reaction we added 1 % TBA and incubated the mixture at 90 °C for 15 min. The absorbance was measured with a UV/Visible spectrophotometer (Jenway 6105, Bibby Scientific Limited, Staffordshire, UK). These results are expressed in $\mu\text{mol L}^{-1}$ erythrocytes.

Determination of GSH and antioxidant enzymes

For GSH determination we used the method of Beutler (26), based on the oxidation of GSH with DTNB (5,5'-dithiobis-2-nitrobenzoic acid). Its concentrations are expressed in $\mu\text{mol L}^{-1}$ of erythrocytes.

Superoxide dismutase (EC 1.15.1.1) activity was determined using the method described by Marklund and Marklund (27). This method is based on pyrogallol oxidation by $\text{O}_2^{\bullet-}$ and its dismutation by SOD. The enzyme activity is expressed as U mL^{-1} of erythrocytes.

For CAT (EC 1.11.1.6) activity measurements we used the method of Beutler (28). The method is based on the rate of H_2O_2 degradation mediated by CAT measured spectrophotometrically at 230 nm in 1 mol L^{-1} Tris-HCl solution (with 5 mmol L^{-1} EDTA, pH 8.0). Absorbance was measured with the Jenway 6105 UV/Visible spectrophotometer and the enzyme activity is expressed in U mL^{-1} of erythrocytes.

Statistical analysis

All analyses were done using the SPSS for Windows, version 13.0 (SPSS Inc., Chicago, IL, USA). All results are expressed as mean±SEM and evaluated using the one-way analysis of variance (ANOVA) test. If there was a significant difference between the groups after applying ANOVA, these groups were compared using Dunnett's multiple comparison tests (control with each treated group). We set statistical significance at $p \leq 0.05$ for all comparisons.

RESULTS AND DISCUSSION

Our findings have confirmed our expectations based on earlier reports about prooxidative effects of nicotine and the antioxidative effects of quercetin and vitamin C (12-14).

Oxidative stress parameters

Erythrocyte concentrations of $O_2^{\bullet-}$, H_2O_2 , and LPO were significantly higher in the nicotine than control group. Co-administration of quercetin and vitamin C greatly reversed nicotine-induced changes in these oxidative stress parameters (Figure 1).

These findings confirm earlier conclusions that nicotine exerts its toxic effects through higher ROS production (3-6, 29). In normal conditions, $O_2^{\bullet-}$ is transformed to H_2O_2 by SOD. If its production increases over the SOD buffering capacity, $O_2^{\bullet-}$ can react with NO to form peroxynitrite (ONOO⁻). Furthermore, in the presence of iron, $O_2^{\bullet-}$ and H_2O_2 can react (Haber-Weiss reaction) to form a highly reactive hydroxyl radical ($^{\bullet}OH$), which can also be produced by peroxynitrite decomposition. Hydroxyl radicals oxidise polyunsaturated fatty acids in biological membranes and

induce the formation of LPO (8). Hence the significantly increased LPO levels in our erythrocytes (Figure 1).

Biochemical test results

Since increased LPO disrupts the normal function or destroys erythrocyte membranes and causes a leakage of the cytoplasmic marker such as LDH (indicator of cell and tissue damage) into circulation (30), we expected to find elevated values of this parameter as well as enhanced activity of AST and ALT, based on earlier findings in blood and various organs (14, 29, 31-34). Indeed, treatment with nicotine significantly increased LDH levels and the activities of ALT, AST compared to control (Table 1). Quercetin and vitamin C reversed these changes toward the control ones and minimised the adverse effects of nicotine (Table 1).

Our study has also demonstrated that by interfering with the metabolism of lipoproteins, nicotine causes a significant increase in total cholesterol, LDLC, and TG levels, whereas the level of HDLC significantly drops (Table 2). This is in line with the study by Sharif et al. (33), who have reported adverse nicotine effects on the lipid profile of adult male mice (*Mus musculus*). Once again, quercetin and vitamin C co-administration have shown their strong protective effects against nicotine toxicity by restoring lipid levels to near normal values (Table 2).

GSH and antioxidant enzymes

The nicotine group showed a significant drop in GSH and antioxidant enzyme activities compared to control, and the treatment with quercetin and vitamin C again reversed these effects (Figure 2).

Table 1 Effects of quercetin and vitamin C on hepatic marker enzyme activities in the serum of nicotine-treated rats (values are expressed as means±SEM of 6 animals)

Parameters	Experimental groups		
	Control	Nicotine	Nicotine + QN + Vit C
ALT (U L ⁻¹)	47.5±1.6	83.2±2.1*	57.4±2.2**
AST (U L ⁻¹)	148.2±5.8	184.3±5.2*	142.6±4.1**
LDH (U L ⁻¹)	625±22.3	1238±65.2*	840±42.3**

QN: quercetin; Vit C: vitamin C; ALT: alanine aminotransferase; AST: aspartate aminotransferase; LDH: lactate dehydrogenase.
*significantly different from control ($p < 0.05$); **significantly different from nicotine group ($p < 0.05$)

Table 2 Effects of quercetin and vitamin C on lipid profile in the serum of nicotine-treated rats (values are expressed as means±SEM of 6 animals)

Parameters	Experimental groups		
	Control	Nicotine	Nicotine + QN + Vit C
TC (mmol L ⁻¹)	1.14±0.06	1.56±0.07*	0.95±0.06**
HDLC (mmol L ⁻¹)	0.82±0.02	0.58±0.03*	0.86±0.05**
LDLC (mmol L ⁻¹)	0.11±0.01	0.37±0.03*	0.20±0.03**
TG (mmol L ⁻¹)	0.18±0.02	0.41±0.04*	0.24±0.05**

QN: quercetin; Vit C: vitamin C; TC: total cholesterol; HDLC: high density lipoprotein cholesterol; LDLC: low density lipoprotein cholesterol; TG: triglycerides. *significantly different from control ($p < 0.05$); **significantly different from nicotine group ($p < 0.05$)

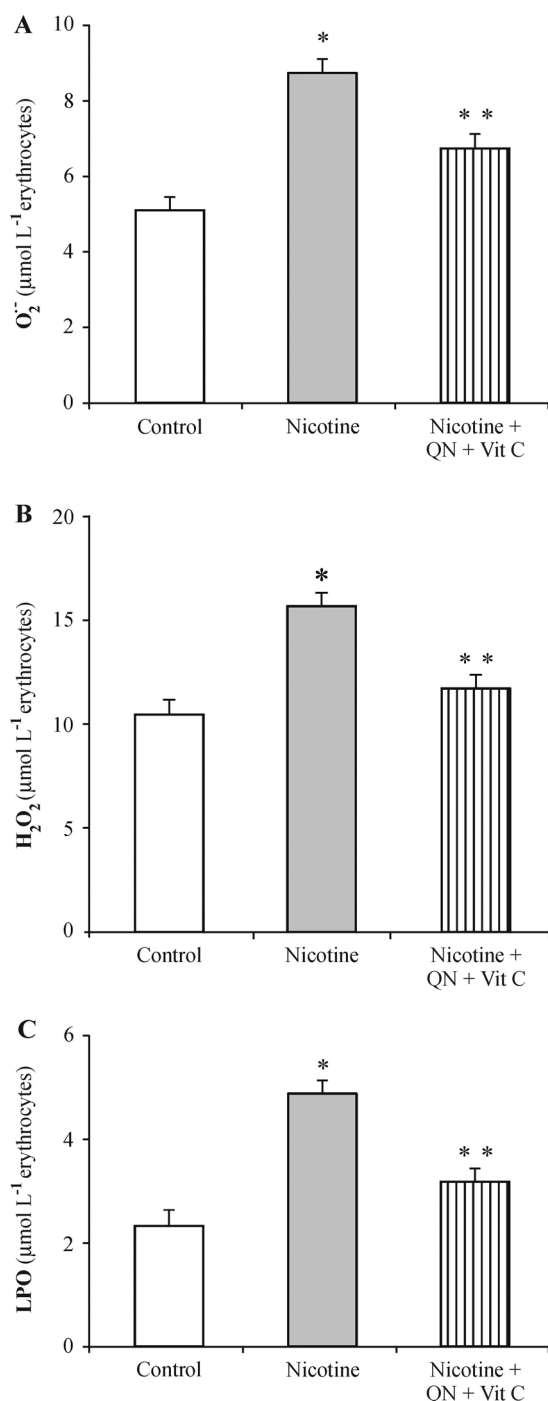


Figure 1 Effects of quercetin and vitamin C on oxidative stress parameters ($O_2^{\bullet-}$, H_2O_2 and LPO) in the blood of nicotine-treated rats (values are expressed as means \pm SEM of 6 animals). QN: quercetin; Vit C: vitamin C; $O_2^{\bullet-}$: superoxide anion radical; H_2O_2 : hydrogen peroxide; LPO: lipid peroxides. *significantly different from control ($p < 0.05$); **significantly different from nicotine group ($p < 0.05$)

The decreased levels of GSH in nicotine-treated rats indicate that GSH protects membrane lipids from oxidation by scavenging free radicals, passing from reduced to oxidized form (GSSG) (14, 31, 35). Oxidative stress in erythrocytes induced by nicotine can inactivate SOD and CAT proteins or affect their synthesis *de novo*, which consequently reduces their activity. Lower SOD activity is due to its reaction with $O_2^{\bullet-}$ to form H_2O_2 and O_2 . H_2O_2 is also a substrate for CAT, whose role is to degrade H_2O_2 into H_2O and O_2 (8), thereby decreasing its activity as we observed in our study. Other studies (31, 35) have also demonstrated the depletion of SOD and CAT in some organs of nicotine-treated rats.

As for the protective effects of the quercetin plus vitamin C combination, they seem to protect phospholipid bilayers by non-covalent bonding, enhancing this way the regeneration of endogenous antioxidants (12). Our results confirm the protective effects of quercetin and vitamin C against nicotine-induced changes in both enzymatic and non-enzymatic antioxidants due to either direct scavenging of ROS or induction of antioxidant enzymes.

There are some limitations to our study: the protective effects of the quercetin plus vitamin C combination was evaluated against nicotine alone, as only one of the many components of tobacco. Therefore, our findings can not be interpreted as simply as to claim that quercetin plus vitamin C would provide the same level of protection against tobacco products in general. Furthermore, we have not compared the protective effects of this combination with quercetin alone, which would probably give a better insight into the mechanisms of their interaction (additive or synergistic). In addition, further research should look into the effects of higher quercetin doses and whether these effects may become prooxidant, as suggested by Heeba and Mahmoud (36). Future research should also be able to elucidate the exact molecular mechanisms of quercetin and vitamin C protection against nicotine or other alkaloids and drugs.

Our findings, however, have practical implications. The combination of quercetin and vitamin C in the selected doses ($40 \text{ mg kg}^{-1} \text{ b.w.}$ and $100 \text{ mg kg}^{-1} \text{ b.w.}$, respectively) seems to sufficiently reinforce the antioxidative defences and diminish the adverse effects of nicotine for us to recommend the adapted doses in persons exposed to nicotine, tobacco smokers in particular.

Conflict of interest

The authors declared that there are no conflicts of interest.

Acknowledgements

This study was supported by the Ministry of Education, Science and Technological Development of Republic of Serbia, grant no. 173041.

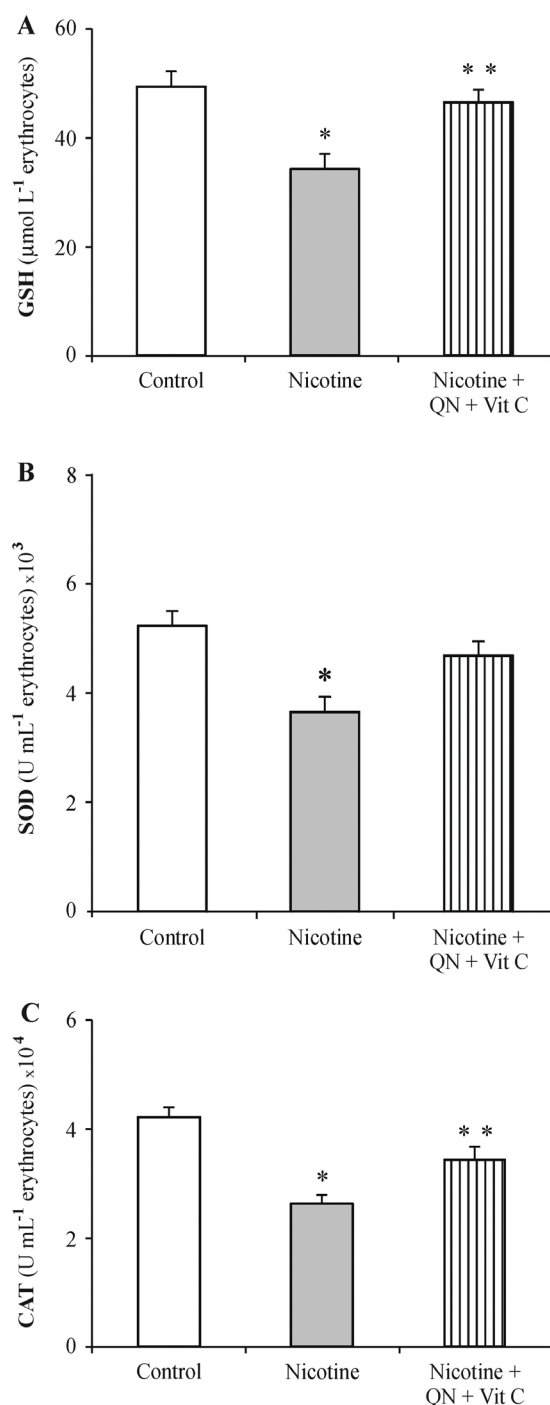


Figure 2 Effects of quercetin and vitamin C on the antioxidative defense system (GSH, SOD, and CAT) in the blood of nicotine-treated rats (values are expressed as means±SEM of 6 animals). QN: quercetin; Vit C: vitamin C; GSH: reduced glutathione; SOD: superoxide dismutase; CAT: catalase. *significantly different from control ($p < 0.05$); **significantly different from nicotine group ($p < 0.05$)

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Zaštitno djelovanje kvercetina i vitamina C protiv nikotinom izazvane toksičnosti u krvi Wistar štakora

Nikotin je potencijalni induktor oksidacijskoga stresa, preko kojega može oštetiti brojne biološke molekule. Cilj našega istraživanja bio je ispitati prooksidacijsko djelovanje nikotina i zaštitno (aditivno ili sinergističko) djelovanje kvercetina i vitamina C u krvi eksperimentalnih životinja te utvrditi može li kombinacija tih antioksidansa biti korisna u kliničke svrhe. *Wistar albino* štakori primali su intraperitonealno injekciju nikotina ($0,75 \text{ mg kg}^{-1}$ po danu) ili fiziološke otopine (kontrolna skupina) ili nikotina s kvercetinom (40 mg kg^{-1} po danu) i vitaminom C (100 mg kg^{-1} po danu) tri uzastopna dana. Četvrtoga dana odredili smo lipidni profil u krvi, jetrene enzime, parametre oksidacijskoga stresa i antioksidacijskoga sustava. U usporedbi s netretiranom kontrolnom skupinom, nikotin je značajno povećao ukupni kolesterol, LDL-kolesterol, trigliceride, jetrene enzime (alanin transaminaze, aspartat transaminaze i laktat dehidrogenaze) i parametre oksidacijskoga stresa (superoksid anion, vodikov peroksid i lipidne perokside), a smanjio HDL-kolesterol, glutation i aktivnosti superoksid dismutaze/katalaze. Kvercetin i vitamin C značajno su utjecali na te vrijednosti u odnosu na skupinu samo s nikotinom. Naši rezultati potvrdili su značajno prooksidacijsko djelovanje nikotina koje može poremetiti redoks ravnotežu i pokazuje da kombinacija kvercetina i vitamina C podržava antioksidacijske obrambene mehanizme s jakim hematoprotekcijskim aktivnostima protiv nikotinom izazvane toksičnosti. Možemo zaključiti da prehrana bogata kvercetinom i vitaminom C može koristiti kao prevencija nikotinom inducirane toksičnosti te da kombinacija tih dvaju antioksidansa može biti korisna u kliničkom oporavku ljudi izloženih nikotinu.

KLJUČNE RIJEČI: *antioksidacijska obrana; duhan; eritrociti; lipidni profil; oksidacijska oštećenja; reaktivne vrste kisika*